SPECTROPHOTOMETRIC DETERMINATION OF BLOOD OXYGEN*

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Gasometric methods for estimating blood oxygen are time-consuming. This consideration often limits the number of observations which can be made in the course of experiments requiring oxygen determinations. In many cases it is an arteriovenous difference which is particularly desired, as in the measurement of cardiac output by the Fick principle, or the estimation of oxygen consumption by human liver, kidney, or brain. The present paper describes a rapid, simple spectrophotometric method for determination of arteriovenous oxygen differences with an accuracy satisfactory for ordinary needs. The absolute oxygen content can also be estimated, subject to error caused by “inactive” hemoglobin. The method is based upon the principle that two well hemolyzed samples of blood drawn nearly simultaneously from the same individual will have substantially the same total hemoglobin content, “inactive” hemoglobin (1), turbidity, and content of other light-absorbing material; and that, consequently, the difference in optical densities of the two samples at an appropriate wave-length will be proportional to the difference in oxyhemoglobin concentration of the two samples. This hypothesis has been tested on samples of blood by comparison of differences in optical density with differences in oxygen content, determined gasometrically by the method of Van Slyke and Neill (2).

The difference in absorption spectra of reduced and oxygenated hemoglobin has been the basis of several methods for determining per cent saturation of blood samples. Much of the basic work in this field was done by Drabkin and his colleagues, who devised a special cell with a short light path, demonstrated the applicability of the Beer-Lambert law to concentrated solutions of hemoglobin and hemoglobin derivatives (3), and measured the per cent of oxygen saturation of undiluted arterial blood samples in man and of circulating arterial blood in the dog (4). Before Drabkin’s work Hall had developed a spectroscopic method for determination of per cent saturation of hemoglobin in dilute solutions (5). This was later extended by the use of an ingenious tonometer to measurements of oxygen saturation in whole blood (6). Early work on

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this subject was also done by Kramer (7) and by Matthes (8). The Millikan oximeter is based on the same principle (9). Several methods in which photoelectric colorimeters are employed have been described. Brinkman and Wildschut (10) constructed an instrument having a neon lamp to provide light, mostly in the range of 600 to 700 m. Using a special cuvette with a short light path, they measured per cent of oxygen saturation in small samples of blood hemolyzed with saponin. The absorption of the sample was compared with its absorption after reduction by Na₂S₂O₄, a procedure used earlier by Drabkin. A similar method was described by Jonxis (11), who used a different instrument and a mercury light source with maximum intensity at 436 m. Von Issekutz (12) employed a photoelectric colorimeter with a filter which provided light in the near infra-red range and diluted his samples with a relatively large volume of an oxygen-free aqueous solution of saponin and ammonia. After an initial reading the diluted sample was either oxygenated or was reduced by Na₂S₂O₄ and the calculation of oxygen content made from the difference in readings. Lowry et al. (13) made use of the Evelyn calorimeter with 490 and 620 m filters and a specially constructed cell with a short light path. Recently Groom et al. (14) has devised a method with a special photometer to make determinations on whole blood contained in transparent plastic tubing.

In most of the methods previously described more or less specialized apparatus is required, particularly cuvettes with a short light path, and small samples of blood were employed. In the present method commercially available equipment and blood samples of 4 to 5 cc. are used.

Equipment and Reagents

1. A Beckman model DU spectrophotometer. The nominal wavelength of 660 m was selected. When the instrument is properly focused and used as described, this requires a slit width of about 0.05 mm., resulting in a spread of 1.5 m on either side of the nominal wave-length, according to the data submitted by the manufacturers. At this wave-length the extinction coefficient of reduced hemoglobin is approximately 6 times that of oxyhemoglobin. We find the extinction coefficient of reduced hemoglobin to be approximately 0.83 (concentration = 1 mm per liter, depth = 1 cm.) and that of oxyhemoglobin approximately 0.13. These results are in reasonable agreement with the curves published by Horecker (15). At this wave-length the 0.5 cm. cuvettes supplied by the manufacturer provide a suitable light path for the density differences usually encountered.

2. A 30 per cent aqueous solution of saponin, made up every other day and centrifuged to obtain a clear solution. Of the brands used, saponin
(practical, Eastman Kodak Company) has given the most satisfactory results. The solution is neutralized to indicator paper by potassium hydroxide.

4. Heparin solution, 10 mg. per cc.
5. A 4 per cent aqueous solution of potassium ferricyanide.
6. A 5 per cent aqueous solution of potassium cyanide.
7. Mercury.
8. Glass syringes, Becton, Dickinson Luer-Lok, 2 cc. and 10 cc., assorted hypodermic needles, and syringe caps (soldered needle hubs).

**Procedure**

Blood samples of 4 to 5 cc. are drawn into oiled, heparinized, 1 10 cc. Luer-Lok syringes which are then capped with a soldered needle hub. After the volume is noted, mercury is added to assist mixing of blood and heparin, and the syringe is stored in ice water. At least one sample is taken for saturation in air and for total hemoglobin determination. If it is suspected that the hemoglobin concentration has changed during the procedure, samples for saturation and total hemoglobin are taken at appropriate times.

When all samples have been collected, they are hemolyzed by 30 per cent saponin solution added in a volume equal to 20 per cent of the blood sample volume. This is conveniently done by removing the cap from the sample syringe, closing the nozzle by a tightly applied single thickness of longitudinally split rubber tubing, perforating the rubber with a needle attached to a 2 cc. syringe containing the saponin solution, and injecting the required amount of saponin into the sample syringe. The procedure is facilitated by working on a table top. The syringe is recapped and immediately shaken so that agitation of the contained mercury will thoroughly mix blood and saponin. Samples are transferred to the open 0.5 cm. cuvettes through a No. 19 needle attached to the syringe, bent at right angles to avoid injecting mercury from the bottom of the syringe and long enough to reach the bottom of the cuvette. Mineral oil is immediately layered over the blood. The density of each sample is read at 660 mμ, with use of the saturated hemolyzed sample as a blank.

The difference in oxygen content between the saturated sample and any other sample is obtained from the empirically determined formula

\[ \text{Oxygen difference (vol. %)} = 7.76 \times \text{optical density difference} + 0.36 \]

Arteriovenous difference may be determined by subtracting saturation-arterial difference from saturationvenous difference, or found directly by

1 Enough heparin solution is used to fill the dead space of the syringe.
measuring the density of the venous sample against that of the arterial sample and multiplying the optical density difference by the factor 7.76. The samples being compared must have the same hemoglobin content, but the arteriovenous difference so determined is otherwise independent of the hemoglobin content.

The absolute oxygen content of each sample, if desired, may be estimated on the basis of total hemoglobin content, together with the difference in oxygen content between the sample and the saturated blood. Total hemoglobin is determined by conversion to cyanmethemoglobin, essentially as described by Drabkin (16). 1 cc. of whole blood is added from an Ostwald-Folin pipette to approximately 150 cc. of distilled water in a 200 cc. volumetric flask. 4 cc. of 4 per cent K₄Fe(CN)₆ are added, followed after 20 minutes by 1 cc. of 5 per cent KCN. The solution is diluted to volume, and its density is read at 540 mμ in 1.0 cm. cuvettes against a reagent blank. Employing gasometrically determined oxygen capacity as a measure of hemoglobin concentration, Myers² has obtained in twelve subjects the value of 11.75 ± 0.23 (standard deviation) for the extinction coefficient (concentration = 1 mm per liter, depth = 1.0 cm.) of cyanmethemoglobin at 540 mμ. With this value, oxygen capacity in volume per cent is obtained from the density reading of the diluted cyanmethemoglobin solution. 1 mm per liter of hemoglobin is equivalent to 1.67 gm. per cent or to an oxygen capacity of 2.27 volumes per cent. The dilution of the sample is 1:200. Accordingly the oxygen capacity in volume per cent is obtained from the photometer reading by the expression

\[
\text{Oxygen capacity} = \frac{\text{optical density} \times 200 \times 2.27}{11.75} = \text{optical density} \times 38.7
\]

The oxygen contents of all the samples may now be estimated by subtracting from the oxygen capacity the oxygen difference between the saturated sample and the individual sample, as calculated from the difference in optical densities,

\[
\text{Oxygen content} = \text{capacity (colorimetric)} - (7.76 \times \text{density} + 0.36)
\]

This determination of absolute oxygen content is subject to whatever error may have been introduced into the colorimetric determination of oxygen capacity by unusual quantities of inactive hemoglobin. The arteriovenous difference, however, is not so affected. In the determination of this quantity by subtraction the value for capacity disappears.

If the hemoglobin concentration of the subject is being changed during the course of a study, the drawing of each sample or set of samples should be accompanied by a further sample for saturation and colorimetric

² Myers, J D., unpublished observations.
oxygen capacity. The above procedure for oxygen content is then followed, with use of the appropriate saturation blank and oxygen capacity for each set.

Aside from obvious sources of error, such as inaccuracies in mixing blood and saponin solution, the most serious error results from failure to obtain satisfactory hemolysis. Before reading, all samples must be inspected for clarity by transmitted light. The clarity of solutions will vary somewhat from subject to subject, depending upon the plasma, but in general the spiral filament of a pocket flashlight should be clearly visible through the hemolyzed blood in the cuvette. Old solutions of saponin may give irregular hemolysis. No difficulty has been encountered in the use of Eastman Kodak saponin freshly made up every other day.

We have had no experience with highly turbid bloods, such as those from subjects with leucemia or pathological lipemia.

In the process of hemolysis, it is essential to mix blood and saponin solutions thoroughly immediately after they are brought together. If the solutions are allowed to layer and stand, a protein precipitate will form in short order at the interface between the blood and the hypertonic saponin solution. We have found no measurable effect for a period of 1 minute, but by 5 minutes a significant cloudiness will develop. All bloods should be hemolyzed together and the densities determined within 30 minutes after hemolysis. Although density differences may be stable for several hours, samples may begin to show significant changes within 40 minutes after hemolysis. With unneutralized saponin, which yields blood saponin mixtures having a pH in the range of 6.2 to 6.5, the color is less stable. Blood samples with a hemoglobin near the normal range will, however, give the same immediate readings with neutralized or unneutralized saponin. For bloods with a hemoglobin content below 8 gm. per cent we have used neutralized 15 per cent saponin solution.

Verification of Method

In order to determine the increment in optical density for a given change in oxygen content of the sample, the following procedure was adopted. 90 to 100 cc. of human blood were drawn from an antecubital vein into an oiled syringe containing enough heparin solution to fill the dead space. Mercury was added to assist in mixing. A portion of the blood was saturated in air. The oxygen contents of the saturated and partially reduced bloods were determined by the method of Van Slyke and Neill (2). Analyses were done in duplicate and were required to check within 0.1 volume per cent. Several mixtures of these bloods containing graded quantities of the oxygenated and partially reduced components were made in oiled 10 cc. syringes of the Luer-Lok type. It was found con-
## Table I

**Determination of Arteriovenous Blood Oxygen Differences**

<table>
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<tr>
<th>Subject No.</th>
<th>Oxygen capacity</th>
<th>Oxygen difference, (saturated) - (sample)</th>
<th>Optical density difference at 660 mλ, (sample) - (saturated)</th>
<th>Subject No.</th>
<th>Oxygen capacity</th>
<th>Oxygen difference, (saturated) - (sample)</th>
<th>Optical density difference at 660 mλ, (sample) - (saturated)</th>
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Regression curve, oxygen difference = 7.76 (optical density difference) + 0.36. Standard deviation from regression, 0.23 volume per cent.

* Depth = 0.5 cm.

Convenient first to place the desired quantity of saturated blood in the mixture syringe, carefully expel air bubbles, and then to add the reduced blood. This was done by injecting through a rubber membrane, as described above. In this way four to six intermediate mixtures were prepared from
the two samples on which gasometric analyses had been done. The oxygen contents of the mixtures were calculated from the proportions of their components. All bloods were then hemolyzed in the manner already described. The saturated sample was used as a blank against which the density of the other samples was read. The data so obtained on 56 original samples and mixtures from nine subjects are presented in Table I. The regression curve calculated from these data together with the individual points appears in Fig. 1. If $Y$ represents difference in oxygen content in volumes per cent between a saturated sample and any other

\[
Y = 7.76X + 0.36
\]

Fig. 1. Each point represents the difference between a partially reduced and a saturated blood sample from a given subject. Oxygen differences are expressed in volumes per cent and were determined on a gasometric basis (see the text). Optical density differences are as read directly from the spectrophotometer at 660 m\u00b5, the saturated sample being used as a blank. There are 56 determinations, representing all data from nine consecutive subjects. The standard deviation from regression is 0.23 volume per cent.

sample from the same subject, and $X$ represents the optical density as read on the machine, the equation for the regression line becomes $Y = 7.76X + 0.36$. The standard deviation from regression is 0.23 volume per cent. Since one is dealing with the difference in oxygen content of two samples, a maximum variation of 0.2 volume per cent would be expected if the individual samples were analyzed gasometrically in duplicate with checks to 0.1 volume per cent.

This agreement with the gasometric method for arteriovenous differences is considered satisfactorily close, particularly in view of the possibilities for error in the verifying procedure described above. These
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include inaccuracies in mixing, addition of oxygen dissolved in the saponin solution, and possible changes in total and inactive hemoglobin during the saturation procedure. Prior to hemolysis, blood was stored in ice for periods up to 4 hours. All readings were completed within 30 minutes after hemolysis.

It will be noted that the line in Fig. 1 does not pass through the origin, but intercepts the ordinate at a height of 0.36 volume per cent. This is apparently the effect of physically dissolved oxygen which is present to a greater extent in the saturated sample than in others and which affects the gasometrically determined oxygen differences but not the optical densities. If the saturation values are neglected and oxygen differences and optical density differences are measured with reference to the prepared mixtures having the greatest oxygen contents (0.7 to 1.5 volumes per cent below capacity), the calculated regression line has the formula, \( Y = 7.80X + 0.14 \). The result agrees with the expectation that dissolved oxygen should exert less effect when the saturated samples are not used.

DISCUSSION

As described above, the method has been in routine use for several months in this laboratory for the determination of blood oxygen contents and arteriovenous differences. This period has covered somewhat more than 300 blood oxygen determinations. In analyzing multiple blood samples the method in our hands has been 5 to 6 times as fast as conventional gasometric procedures.

Of particular interest is the finding that determinations by this method have shown remarkable constancy in per cent oxygen saturation of arterial samples successively drawn from resting normal subjects over a period of 1 to 2 hours. In our experience fluctuations in this measurement in normal subjects are occasionally seen with gasometric techniques and usually indicate technical difficulties.

It should be emphasized that the method depends upon measuring optical density differences between blood samples having the same hemoglobin concentration.

The hemoglobin concentration of normal subjects can undergo very significant changes within a few hours in the course of ordinary daily activities (17). It can be altered markedly in a brief time by changes

\footnote{A few calibrations have indicated that the volume marks on Becton, Dickinson Luer-Lok syringes are sufficiently accurate for the present purpose.}

\footnote{Analysis of the saponin solution has yielded values of approximately 0.4 volume per cent of oxygen. The effect of this quantity, mixed with 5 times its volume of blood and added to all the samples, has been neglected.}
in body position and by exercise (18). It may be affected by drugs, such as epinephrine. It varies with local factors, such as venous stasis. In using the method these variations must be kept in mind. When the hemoglobin concentration changes, a fresh sample for saturation must be taken, together with the samples on which oxygen contents are to be determined. Similarly, arteriovenous differences can be determined directly only by comparing samples which have the same hemoglobin concentration.

In adopting such a method, it is apparent that the factors for one’s own spectrophotometer as well as the general technique should be checked by gasometric analysis.

SUMMARY

1. A spectrophotometric method is described for the determination of arteriovenous blood oxygen differences and the estimation of blood oxygen contents.

2. The accuracy is satisfactory for most purposes. In determination of arteriovenous differences which have been checked by gasometric analysis, the method has yielded a standard deviation from regression of 0.23 volume per cent.

3. The advantage of the method lies in the speed with which multiple samples can be handled.

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